FLOCCULATION WITH DIVALENT SALT

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation in part of a US application No. 10/463,939, filed June 18, 2003, which claims, under 35 U.S.C. 119, priority from Danish application no. PA 2002 00944 filed June 20, 2002, and the benefit of U.S. provisional application no. 60/390,308, filed June 21, 2002, the contents of which are fully incorporated herein by reference.

TECHNICAL FIELD

The present invention relates to a simple and effective method for flocculation of a micro-organism, producing a glycosaminoglucan of interest, such as hyaluronic acid from a fermentation broth.

BACKGROUND ART

Hyaluronic acid (HA) is a mucoid polysaccharide of biological origin, which is widely distributed in nature. For example, it is known that hyaluronic acid is present in animal tissues such as umbilical cord, synovial fluid, vitreous humor, rooster comb, and various connective tissues such as skin and cartilage.

Hyaluronic acid, at high molecular weight, is viscous and able to maintain a jelly state, acting as a lubricant, preventing the invasion of bacteria and retaining water.

Due to the above mentioned properties, hyaluronic acid has technical and cosmetic uses, e.g., hyaluronic acid is able to retain the tonicity and elasticity of the skin. Pharmaceutical use of hyaluronic acid is widely described in the literature, e.g., as an eye vitreous or as a supportive medium in ophthalmic surgery or as a joint fluid replacement.

Thanks to its highly hydrophilic nature, hyaluronic acid may also be used in cosmetic products such as lotions and creams.

Hyaluronic acid may be extracted and purified from e.g. umbilical cords, from rooster combs or from group A and C Streptococci.

The hyaluronic acid may also be produced by recombinant production in a host cell such as a bacterial cell or a fungal cell.

When hyaluronic acid is produced by fermentation of a micro-organism, the cell mass is conventionally removed by filtration or centrifugation at high dilution. Flocculation allows a more efficient cell removal and also the use of a wider range of cell removal techniques (e.g. drum filtration, dead end filtration, centrifugation etc). However, flocculation chemicals can adversely affect the product or impede down stream processing strategies.

SUMMARY OF THE INVENTION

We have found that it is possible to flocculate a micro-organism, producing a glycosaminoglucan of interest, in a very efficient way: Addition of a divalent salt has been shown to create small flocs which can be easily separated from the cell broth whilst not reducing product yield, or impeding subsequent down stream operations.

The present invention provides a method of flocculating a micro-organism and/or removing high molecular weight contaminants from a fermentation broth, comprising adding a divalent salt to the fermentation broth comprising a glycosaminoglucan of interest after which the micro-organism and/or the high molecular weight contaminants are removed, wherein said micro-organism produces the glycosaminoglucan of interest.

DETAILED DISCLOSURE OF THE INVENTION

The present invention provides a method for flocculating a micro-organism, producing a glycosaminoglucan of interest, from a fermentation broth comprising adding a divalent salt to the fermentation broth whereafter the micro-organism is removed.

Glycosaminoglucans

According to the invention a glycosaminoglucan may be any carbohydrate polymer having a molecular weight of at least 700 Daltons; preferably a molecular weight of at least 10,000 Daltons; more preferably a molecular weight of at least 20,000 Daltons; even more preferably a molecular weight of at least 30,000 Daltons.

Preferred glycosaminoglycans are hyaluronic acid, chondroitin sulphate, chondroitin (non-sulphated), heparin, heparin sulphate, dermatan sulphate, and keratin sulphate. Hyaluronic acid is constituted by alternating and repeating units of D-glucoronic acid and N-acetyl-D-glucosamine, to form a linear chain having a molecular weight of up to 15,000,000 Daltons.

Preferred glycosaminoglucans according to the invention are glycosaminoglucans having a molecular weight of from 700 Daltons to 15,000,000 Daltons.

It is to be noted that the term "hyaluronic acid" in the present application and claims may mean indifferently hyaluronic acid in its acidic form or in its salt form such as for example sodium hyaluronate, potassium hyaluronate, magnesium hyaluronate, calcium hyaluronate, or others.

Fermentation broth

The glycosaminoglucan may be obtained from any fermentation broth. The glycosaminoglucan may furthermore be one which is producible by a method comprising cultivating a host cell.

The host cell may preferably be a micro-organism. The micro-organism may be a unicellular micro-organism, *e.g.*, a prokaryote, or a non-unicellular micro-organism, *e.g.*, a eukaryote. Useful unicellular cells are bacterial cells such as gram positive bacteria including, but not limited to, a *Bacillus* cell, *e.g.*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus firmus*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus stearothermophilus*, *Bacillus subtilis*, and *Bacillus thuringiensis*; or a *Streptomyces* cell, *e.g.*, *Streptomyces lividans* or *Streptomyces murinus*, or gram negative bacteria such as *E. coli* and *Pseudomonas* sp. In a preferred embodiment, the bacterial host cell is a *Bacillus lentus* cell, a *Bacillus licheniformis* cell, a *Bacillus stearothermophilus* cell or a *Bacillus subtilis* cell. Mutant *Bacillus subtilis* cells particularly adapted for recombinant expression are described in WO 98/22598.

The host cell may be a eukaryote, such as a mammalian cell, an insect cell, a plant cell or a fungal cell. Useful mammalian cells include Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, COS cells, or any number of other immortalized cell lines available, *e.g.*, from the American Type Culture Collection. The transformation method, selectable marker gene and any other parts of the expression construct may be chosen from those well known and available to one skilled in the art.

The host cell may be a fungal cell. "Fungi" as used herein includes the phyla Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota as well as the Oomycota and all mitosporic fungi. Representative groups of Ascomycota include, *e.g.*, *Neurospora*, *Eupenicillium* (=*Penicillium*), *Emericella* (=*Aspergillus*), *Eurotium* (=*Aspergillus*), and the true yeasts listed below. Examples of Basidiomycota include mushrooms, rusts, and smuts. Representative groups of Chytridiomycota include, *e.g.*, *Allomyces*, *Blastocladiella*, *Coelomomyces*, and aquatic fungi. Representative groups of Oomycota include, *e.g.*, Saprolegniomycetous aquatic fungi (water molds) such as *Achlya*. Examples of mitosporic fungi include *Aspergillus*, *Penicillium*, *Candida*, and *Alternaria*. Representative groups of Zygomycota include, *e.g.*, *Rhizopus* and *Mucor*.

The fungal host cell may be a yeast cell. "Yeast" as used herein includes ascosporogenous yeast (Endomycetales), basidiosporogenous yeast, and yeast belonging to the Fungi Imperfecti (Blastomycetes). The ascosporogenous yeasts are divided into the families Spermophthoraceae and Saccharomycetaceae. The latter is comprised of four subfamilies, Schizosaccharomycoideae (*e.g.*, genus *Schizosaccharomyces*), Nadsonioideae, Lipomycoideae, and Saccharomycoideae (*e.g.*, genera *Kluyveromyces*, *Pichia*, and *Saccharomyces*). The basidiosporogenous yeasts include the genera *Leucosporidim*, *Rhodosporidium*, *Sporidiobolus*, *Filobasidium*, and *Filobasidiella*. Yeast belonging to the

Fungi Imperfecti are divided into two families, Sporobolomycetaceae (*e.g.*, genera *Sporobolomyces* and *Bullera*) and Cryptococcaceae (*e.g.*, genus *Candida*).

In another embodiment, the fungal host cell is a filamentous fungal cell. "Filamentous fungi" include all filamentous forms of the subdivision Eumycota and Oomycota. The filamentous fungi are characterized by a mycelial wall composed of chitin, cellulose, glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligately aerobic. In contrast, vegetative growth by yeasts such as *Saccharomyces cerevisiae* is by budding of a unicellular thallus and carbon catabolism may be fermentative. In a more preferred embodiment, the filamentous fungal host cell is a cell of a species of, but not limited to, *Acremonium*, *Aspergillus*, *Fusarium*, *Humicola*, *Mucor*, *Myceliophthora*, *Neurospora*, *Penicillium*, *Thielavia*, *Tolypocladium*, and *Trichoderma*.

Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*.

The micro-organism producing the glycosaminoglucan of interest is cultivated in a nutrient medium suitable for production of the glycosaminoglucan using methods known in the art. For example, the micro-organism may be cultivated by shake flask cultivation, small-scale or large-scale fermentation (including but not limited to continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (*e.g.*, in catalogues of the American Type Culture Collection).

<u>High molecular weight contaminants / impurities</u>

Non-glycosaminoglucan materials in the same or higher molecular weight range as the glycosaminoglucan of interest itself, such as, but not limited to: levan, glutamic acids, nucleic acids, cellular debris and components etc.

By using the method of the invention it is possible to remove high molecular weight contaminants/impurities; it is also possible to remove these impurities from cell free preparations of the glycosaminoglucan of interest.

<u>Flocculation</u>

The method of the invention may be applied to an untreated fermentation broth or to a fermentation broth that has first been subjected to, but not limited to, e.g., a pH

adjustment, a temperature adjustment, and/or a water dilution.

According to the invention it has been found that an addition of a divalent salt to the fermentation broth results in a flocculation with small flocs which can be easily separated from the cell broth.

The evaluation of floc sizes is normally done by comparing the observed flocs with a photograph of various floc sizes.

It will often be an advantage to dilute the fermentation broth with water before, simultaneously or after the addition of the divalent salt. Depending on the yield of the glycosaminoglucan and the wanted viscosity a dilution with 100-500% (w/w) of water of the fermentation broth will be appropriate; preferably a dilution with 100-400% (w/w) of water of the fermentation broth.

It will often be an advantage to heat the fermentation broth before, simultaneously or after addition of the divalent salt, or to heat the fermentation broth before, simultaneously or after the dilution depending on the yield of the glucosaminoglucan of interest and the wanted viscosity. The fermentation broth is preferably heated to a temperature above 10°C, in particular to a temperature between 30°C and 90°C, preferably to a temperature between 40°C and 70°C.

It will often be an advantage to adjust the pH of the fermentation broth before, simultaneously or after addition of the divalent salt and or the dilution. Preferably the fermentation broth is adjusted to a pH between 6.5 and 14, in particular to a pH between 7.5 and 8.5.

Divalent salts

According to the invention a surprisingly good flocculating agent is a divalent salt, in particular a calcium and/or a magnesium salt. A preferred divalent salt is a phosphate, a sulphate or a chloride, e.g., calcium chloride. A preferred embodiment is a calcium salt.

The minimum quantity of a divalent salt which will flocculate is dependent upon the micro-organism of the fermentation broth, the constituents of the fermentation broth and the type and the concentration of the salt itself. It should also be noted that overdosing of the divalent salt may reduce the yield. The divalent salt should be added to at least 0.1 g of divalent salt per gram of dry mass of the micro-organism of the fermentation broth, preferably the divalent salt may be added in a range of 0.5-25 g of divalent salt per gram of dry mass of the micro-organism of the fermentation broth, more preferably the divalent salt may be added in a range of 0.5-15 g of divalent salt per gram of dry mass of the micro-organism of the fermentation broth, most preferably the divalent salt may be added in a range of 0.5-8 g of divalent salt per gram of dry mass of the micro-organism of the fermentation broth, in particular

the divalent salt may be added in a range of 0.5-3.5 g of divalent salt per gram of dry mass of the micro-organism of the fermentation broth.

The dosage of the divalent salt is typically done either in-line or in a mixing tank or by any other method known in the art.

It may be an advantage, in addition to the divalent salt, to add one or more flocculating agents such as an aluminate, e.g., NaAlO2, or a cationic or an anionic polymer.

It may also be an advantage, in addition to the divalent salt, to add activated carbon.

After addition of the divalent salt, and optionally the other flocculating agents and/or the activated carbon, the micro-organisms are removed by methods known in the art such as, but not limited to, filtration, e.g. drum filtration, membrane filtration, filterpress, dead end filtration, cross-flow filtration, or centrifugation.

Subsequent downstream operations

The resulting glycosaminoglucan may be further isolated by methods known in the art. For example, the glycosaminoglucan may be recovered by conventional procedures including, but not limited to, further filtration, extraction, spray-drying, evaporation, precipitation or crystallization. The isolated glycosaminoglucan may then be further purified and/or modified by a variety of procedures known in the art including, but not limited to, chromatography (*e.g.*, ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (*e.g.*, preparative isoelectric focusing), differential solubility (*e.g.*, ammonium sulfate precipitation), or extraction.

The invention is further illustrated in the following examples which are not intended to be in any way limiting to the scope of the invention as claimed.

EXAMPLE 1

HA Flocculation Example

Source: GMM *B. subtilis* containing the hyaluronic acid cassette produced as described in WO 03/054163.

Fermentation:

We used a 1500 litre scale; used a defined medium employing inorganic nitrogen as the sole nitrogen source and sucrose dosing as the carbon source.

Final dry cell mass in the fermentation broth was approximately 15 to 20 gl^{-1} on a dry cell weight basis. (Difficult to assess precisely due to high viscosity of the broth).

Hyaluronic acid in the fermentation broth was approximately 10 gl^{-1} with an average molecular weight of approximately 1,400,000 Da. (Measured by ELISA and GPC-MALLS

respectively).

Cell Separation:

Dilution: 200% (1 in 3) dilution with water

Temperature: 10°C

pH 7.0

Flocculation: 2 wt % Calcium chloride relative to the culture broth, or approximately 1.0 to 1.3 g of Calcium chloride per g of dry cells.

Cell removal:

The flocculated cells were easily removed from the hyaluronic acid containing broth under normal equipment operating conditions and fluxes with little loss of yield by one or more of the following methods:

a: Dead end "nutch " filtration. Normal operating conditions.

b: Centrifugation at bench scale in 50 ml centrifuge tubes. Normal operating conditions.

c: Drum filtration (2.5 m^2 area). Normal operating conditions.

d: Centrifugation at pilot scale. Normal operating conditions.

We tested (a)-(d), and in each case we obtained a remarkably clear, straw coloured liquid containing the majority of the hyaluronic acid in the original fermentation broth.

The ease of separation would suggest that cell separation would also be improved on other cell separation equipment (e.g. filter press, microfiltration, etc).

EXAMPLE 2

HA Flocculation Example pilot scale, pH 6.5

Source: GMM *B. subtilis* containing the hyaluronic acid cassette produced as described in WO 03/054163.

Fermentation:

We used a 1500 litre scale; used a defined medium employing inorganic nitrogen as the sole nitrogen source and sucrose dosing as the carbon source.

Final dry cell mass in the fermentation broth was approximately 10 to 15 gl^{-1} on a dry cell weight basis. (Difficult to assess precisely due to high viscosity of the broth).

Hyaluronic acid in the fermentation broth was approximately 12 gl^{-1} with an average molecular weight of approximately 1,000,000 Da. (Measured by ELISA and GPC-MALLS respectively).

Cell Separation:

Dilution: 300% (1 in 4) dilution with water

Temperature 35 °C

Flocculation: 2 wt % Calcium chloride relative to the culture broth or 1.3 to 2.0 g of Calcium chloride per g of dry cells.

pH 6.5

Cell removal:

The flocculated cells were easily removed from the hyaluronic acid containing broth under normal equipment operating conditions and fluxes with little loss of yield by one or more of the following methods:

a: Dead end "nutch " filtration. Normal operating conditions.

b: Centrifugation at bench scale in 50 ml centrifuge tubes. Normal operating conditions.

c: Drum filtration (2.5 m^2 area). Normal operating conditions.

d: Centrifugation at pilot scale. Normal operating conditions.

We tested (a)-(d), and in each case we obtained a remarkably clear, yellow straw coloured liquid containing the majority of the hyaluronic acid in the original fermentation broth.

The ease of separation would suggest that cell separation would also be improved on other cell separation equipment (e.g. filter press, microfiltration, etc).

EXAMPLE 3

HA Flocculation Example process scale, pH 8

Source: GMM *B. subtilis* containing the hyaluronic acid cassette produced as described in WO 03/054163.

Fermentation:

We used a 20 000 litre scale; used a defined medium employing inorganic nitrogen as the sole nitrogen source and sucrose dosing as the carbon source.

Final dry cell mass in the fermentation broth was approximately 8 to 10 gl^{-1} on a dry cell weight basis.

Hyaluronic acid in the fermentation broth was approximately 10 gl^{-1} with an average molecular weight of approximately 1,000,000 Da. (Measured by ELISA and GPC-MALLS respectively).

Cell Separation:

Dilution: 400% (1 in 5) dilution with water

Temperature 45°C

pH 8.0

Flocculation: 2 wt % Calcium chloride relative to the culture broth or approximately 2.0 to 2.5 g of Calcium chloride per g of dry cells.

Cell removal:

The flocculated cells were easily removed from the hyaluronic acid containing broth under normal equipment operating conditions and fluxes with little loss of yield by one or more of the following methods:

a: Dead end "nutch " filtration. Normal operating conditions.

b: Centrifugation at bench scale in 50 ml centrifuge tubes. Normal operating conditions.

c: Drum filtration (process scale). Normal operating conditions.

d: Filter press (pilot scale). Normal operating conditions.

We tested (a)-(d), and in each case we obtained a remarkably clear, straw coloured liquid containing the majority of the hyaluronic acid in the original fermentation broth. High molecular weight contaminants (as assessed by GPC analysis before and after treatment as well as by enzymatic digestion) were comprehensively removed.

The ease of separation would suggest that cell separation would also be improved on other cell separation equipment (e.g. microfiltration, etc). Operation at this dilution and temperature reduced viscosity such that flux rates were higher than in examples 1 or 2. Further, the cellular and other contaminant clearance (measured by cell counts and turbidity) were also greater than in examples 1 or 2.

EXAMPLE 4

HA Flocculation Example pilot scale, contaminant removal experiment

Source: GMM *B. subtilis* containing the hyaluronic acid cassette produced as described in WO 03/054163.

Fermentation:

We used a 1500 litre scale; used a defined medium employing inorganic nitrogen as the sole nitrogen source and sucrose dosing as the carbon source.

Final dry cell mass in the fermentation broth was approximately 8 to 10 gl^{-1} on a dry cell weight basis.

Hyaluronic acid in the fermentation broth was approximately 10 gl^{-1} with an average molecular weight of approximately 1,000,000 Da. (Measured by ELISA and GPC-MALLS respectively).

Cell Separation:

Dilution: 300% (1 in 4) dilution with water

Temperature 15 to 60°C

pH 5.0 to 10.0

Flocculation: 2 wt % Calcium chloride relative to the culture broth or approximately 2.0 to 2.5 g of Calcium chloride per g of dry cells.

Cell removal:

The flocculated cells were easily removed from the hyaluronic acid containing broth under normal equipment operating conditions and fluxes with little loss of yield by a dead end "nutch " filtration. Normal operating conditions. A control without calcium chloride failed to filter or to clarify the treated culture broth.

Investigation of the collected filtrates by GPC-MALLS, enzymatic digestion and other analyses revealed that the degree of high molecular weight contaminant removal could be controlled by pH with the highest degree of removal being below pH 5.5 and at pH > 7.5; pH 8 being the preferred value for process operations. Degree of high molecular weight contaminant removal was found to be independent of temperature or dilution.